vehicle. Sub-cellular localization of the CX26 protein in rescued animals was normal and apparent in support cells throughout the cochlea, and these animals showed increased numbers of surviving hair cells. These preclinical results with AAV-mediated GJB2 gene therapy support the use of OTO-825 as a clinical candidate to treat congenital hearing loss caused by GJB2 deficiency.

132. AAV9 Mediated Delivery of PUF RNA Targeting System Corrects Molecular and Functional Defects in Amyotonic Dystrophy Type 1 Mouse Model

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Myotonic dystrophy type I (DM1) is a multisystemic autosomaldominant inherited disorder caused by CUG microsatellite repeat expansions (MREs) in the 3' untranslated region (UTR) of the DMPK mRNA. Previously, we showed that a CRISPR-based RNA-targeting gene therapy has the potential to eliminate toxic RNAs expressed from repetitive tracts in DM1 in primary patient cells and a DM1 mouse model. We engineered a novel PUF RNA-binding protein system derived from the naturally-occurring human PUM1 protein linked with an RNA endonuclease (E17) derived from human ZC3H112A to target and cleave expanded DM1-related CUG repeats. AAV9-packaged PUF-E17 was delivered via intramuscular (IM) and intravenous (IV) injections to adult (8-weeks old) HSALR DM1 mice. We report sustained PUF-E17 expression at 4- and 12-weeks post injection. Consequently, we observed ~70% decrease in CUG nuclear foci by RNA-FISH, ~50% decrease in the levels of CUG-containing RNA, and efficient reversal of DM1-related mis-splicing to near wildtype levels 4 weeks post injection. We also compared RNA-targeting Cas9, Cas13d, and PUF-E17 systems in vivo and show comparable efficacy in elimination of toxic CUG repeats. Finally, we show that PUF-E17 treatment is safe in HSALR mice giving us multiple safe treatment options for this neuromuscular disorder.

133. Intracorneal and Sequential Contralateral Dosing of AAV-opt-ARSB Reverses MPS VI Corneal Clouding

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Mucopolysaccharidosis VI (MPS VI) is a rare, autosomal recessive lysosomal storage disease caused by mutations in ARSB which encodes arylsulfatase B. Corneal blindness in MPS VI patients compromises life quality and there is no effective treatment. The purpose of these studies was to validate an AAV gene therapy for MPS VI corneal clouding. Initially, the human ARSB ORF was compared to codon optimized cDNA sequences to identify the variant most productive for arysulfatase B activity in vitro (termed opt-ARSB). MPS VI felines, homozygous for a null ARSB mutation, presented diffuse mild corneal clouding and peripheral corneal vascularization OU with no retinal dysfunction. At 21 weeks of age, $ARSB^{-/-}$ felines were unilaterally administered AAV8-opt-ARSB ($1e^9$ viral genomes (vg)) via intracorneal injection. Clinical ophthalmic examinations (slit lamp biomicroscopy, tonometry, pachymetry, ophthalmoscopy, corneal OCT) were performed throughout the study and corneal confocal microscopy was employed at the experimental conclusion. Following the AAV8-opt-ARSB injection, the pre-existing corneal clouding cleared in the central 85-95% of the cornea within 3 weeks. In contrast, the severity of corneal disease progressed in the contralateral vehicle treated cornea. Eight weeks following the initial vector injection, the vehicle treated $ARSB^{-/-}$ cornea was administered AAV8-opt-ARSB ($1e^9$vg). Within 2 weeks of this sequential injection clearance of the storage disease was observed. All AAV8-opt-ARSB injections to the corneal stroma were well tolerated and all vector treated $ARSB^{-/-}$ corneas exhibited >85% clarity until the humane endpoint (90 days following the sequential AAV8-opt-ARSB administration). Vector biodistribution analyses, vector derived cDNA abundance, histological analyses, as well as serum AAV8 neutralizing antibody titers will also be presented. The collective data following intracorneal injection of AAV8-opt-ARSB in MPS VI felines, in conjunction with the large data set of a similar approach for MPS I corneal disease, suggest that intracorneal AAV gene therapy is safe and effective to reverse corneal clouding observed in several lysosomal storage diseases. Importantly, this is the first study to demonstrate in any disease model that sequential AAV vector dosing of the cornea, is safe and remarkably therapeutic with no immunological or adverse consequences.

134. Efficacy and Biodistribution of Anc80-RKhRPGRIP1 Gene Therapy in a Mouse Model of Rpgrip1 Deficiency and in Non-Human Primate

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Introduction: Mutations in the *RPGRIP1* (retinitis pigmentosa GTPase regulator interacting protein 1) gene are a cause of LCA6 (Leber congenital amaurosis 6) and account for ~3-5% of total autosomal recessive blindness. RPGRIP1 plays a vital role in the development and maintenance of photoreceptors due to its role in the

trafficking of proteins along the connecting cilium. A null mutation in the *RPGRIP1* gene leads to progressive and irreversible blindness typically during childhood. Other mutations are associated with a later onset of disease. We are currently investigating RPGRIP 1 gene therapy in a *Rpgrip1* knockout mouse model. Here, we describe biodistribution studies in wildtype (WT) mice and non-human primates (NHP) and efficacy studies in *Rpgrip1* knockout mice. **Methods:** An initial biodistribution and dose response study was conducted using five doses (between 6.76

x 10^9 - 8.02 x 10^{11} vg/eyetotal dose) in 4-6 week-old WT C57BL/6J mice and analyzed for vector biodistribution using qPCR and protein expression using an ECL (Enhanced Chemiluminescence) assay. The extent of Anc80-hRK-RPGRIP1 transduction (at doses 4.5 x 10^{10} and 4.5 x 10^{11} vg/eye in NHP and 2e9 vg/eye in mouse) was determined using *in situ* hybridization (ISH) probes against RPGRIP1 DNA and mRNA in mouse and NHP. To assess efficacy of RPGRIP1 gene replacement therapy in PND21 *Rpgrip1*$^{-/-}$ mice, an Anc80 AAV vector containing a human rhodopsin kinase promotor driving the expression of RPGRIP1 was injected subretinally into one eye of each *Rpgrip1*$^{-/-}$ mouse at doses of 7.36 x 10^{10}, 2.43 x 10^{11} or 8.02 x 10^{11} vg/eye. Each contralateral eye was injected with vehicle as a control. At 12- and 24-weeks post-subretinal injections, the full field electroretinograms (ERGs) and optical coherence tomography (OCT) were recorded for measuring retinal function and structure, respectively. *Rpgrip1*$^{-/-}$ mice injected subretinally with Anc80-hRK-RPGRIP1 were analyzed for RPGRIP1 protein localization using immunohistochemistry along with other markers, such as acetylated tubulin and rootletin. **Results:** Subretinal delivery of Anc80-hRK-RPGRIP1 in WT C57BL/6J resulted in a dose-dependent increase in AAV vector distribution and human RPGRIP1 protein expression. Similarly, NHP retinas showed a dose dependent increase in Anc80-hRK-RPGRIP1 transduction and mRNA expression analyzed using ISH assay. ERG and OCT analysis showed improved preservation of photoreceptor/retinal function and improved photoreceptor survival in the treated eyes compared to untreated eyes 12 weeks after subretinal injection in *Rpgrip1*$^{-/-}$ mice. Subretinal delivery of the Anc80-hRK-RPGRIP1 in *Rpgrip1*$^{-/-}$ mice confirmed human RPGRIP1 protein was expressed specifically in photoreceptors and localized correctly in the connecting cilia. **Conclusions:** The present study demonstrates improvement in photoreceptor survival and function after subretinal delivery of Anc80-hRK-RPGRIP1. Efficacy was associated with the proper localization of human RPGRIP1 protein at the connecting cilium.

135. Bicistronic AAV Gene Therapy for Tay-Sachs and Sandhoff Diseases

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Tay-Sachs and Sandhoff diseases (TSD, SD) are neurodegenerative diseases, with similar clinical manifestations, caused by mutations in alpha or beta subunit of enzyme Hexosaminidase (Hex) respectively. Deficiency of Hex results in GM2 ganglioside storage and neuronal death. The Hex isozyme (Hex A) that degrades GM2 in humans is a heterodimer of alpha and beta subunits; therefore, co-expression of both subunits is necessary for restoration of Hex function. Here we describe the therapeutic efficacy of a single, bicistronic AAV9 vector construct that delivers both Hex subunits simultaneously in a naturally occurring sheep model of TSD. TSD sheep were injected with 1E14 vg total intravenously (IV, n=5, 5 days of age) or via the cerebrospinal fluid (CSF, n=5, 3-6 weeks of age). Sheep treated intravenously survived to 18±5 months of age and CSF treated sheep are all ongoing with the oldest now more that 3 years of age. Untreated TSD sheep reach the humane endpoint at ~ 9 month of age. GM2 ganglioside levels in CSF of 1- and 2-year-old animals treated by CSF administration were completely normalized ($P<0.0009$ vs TSD; $P<0.88$ vs normal), however, the GM2 ganglioside levels in the IV cohort were between that in untreated TSD and normal sheep. Both IV and CSF AAV treated TSD sheep exhibit marked attenuation of neurologic disease as well as normal cognition as measured by maze testing. Magnetic resonance spectroscopy (MRS) of treated sheep thalamus at ~2 years of age showed normalization of markers of neuronal health, myelination and metabolism in the CSF cohort and a slight improvement in neuronal health in the IV cohort. Diffusion tensor imaging (DTI) analysis demonstrates the decrease in microstructural integrity and increase in water diffusion in white matter of TSD brain which is a consistent finding in neurodegenerative diseases. IV treatment partially corrected these changes in some brain regions. IV treatment resulted in Hex A activity in spinal cord at ~ 70% and 100% of normal level in cervical and lumbar sections, ~ 20% in cerebellum and brainstem and lower levels in other brain regions. GM2 content in caudal aspect of the brain correlates (>80%) with Hex A activity. HexA level in skeletal muscle was ~90% of normal, but levels were lower in skeletal and other peripheral organs and tissues. Further studies are ongoing with both cohorts. Non-invasive imaging and GM2 content analysis on CSF have been routinely performed on long term CSF cohort (~ 3 years of age) to monitor the therapeutic efficacy using non-invasive biomarkers of the disease and provide data to further aid clinical translation. To enable early assessments of biomarkers and biochemistry in postmortem tissues after CSF administration, a short-term CSF treatment cohort has been initiated (n=4, endpoint ~6 months of age). These data show promise for a minimally invasive treatment for TSD and SD using this bicistronic

vector construct which will provide the basis for submission of a pre-IND application.

136. Gene Therapy Rescues Cone and Rod Function in a Pre-Clinical Model of *CDHR1*-Associated Retinal Degeneration through Restoration of Photoreceptor Outer Segments

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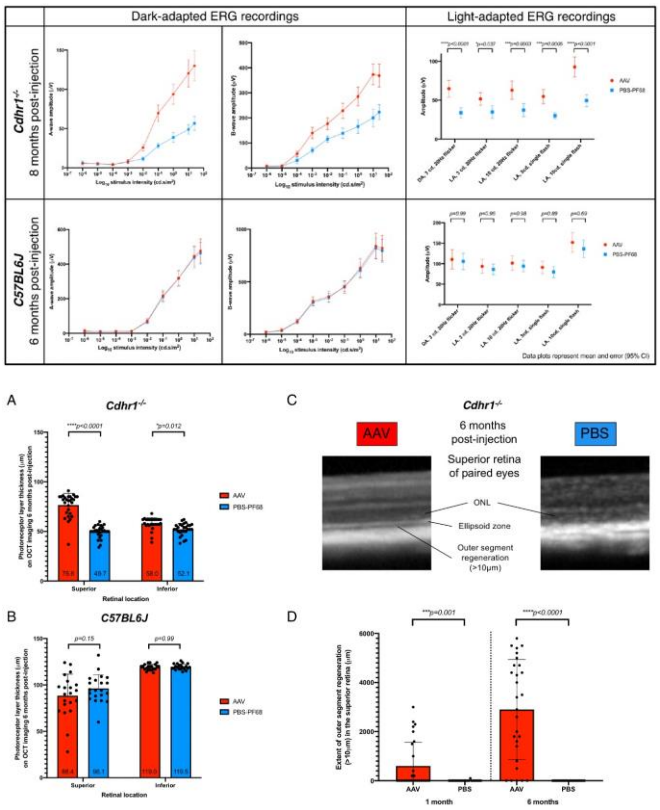
Purpose To evaluate the efficacy and safety of retinal gene therapy in a pre-clinical model of *CDHR1*-associated retinal degeneration - an as yet untreatable, blinding disorder characterised by progressive cone and rod photoreceptor degeneration. **Methods** *Cdhr1*$^{-/-}$ *(n=28)* and *C57BL6J* control mice *(n=23)* underwent paired sub-retinal injections of AAV8.GRK1.CDHR1 ($1.5x10^8$) and PBS vehicle control in the fellow eye at 4 weeks of age. Dark- and light-adapted electroretinography (ERG) was undertaken at 2, 4, 6 and 8 months post-injection. Photoreceptor layer thickness measurements were compared using optical coherence tomography (OCT) imaging at 1 and 6 months post-injection. **Results** In *Cdhr1*$^{-/-}$ mice, sub-retinal delivery of AAV8.GRK1.

CDHR1 rescued A-wave amplitudes (*p<0.0001* at all time points) and B-wave amplitudes (*p<0.0001* from 6 months) on the dark-adapted ERG luminance series when compared with PBS-injected control eyes **(Fig.1)**. Light-adapted flicker ERG amplitudes were greater in AAV-treated eyes at 8-months post-injection (*p<0.0001*). Sub-retinal injection of AAV8.GRK1.CDHR1 preserved photoreceptor layer thickness in the superior retina versus PBS-injected control eyes at 6-months post-injection (mean: 76.6µm versus 49.7µm; *p<0.0001;* **Fig.2**). OCT changes consistent with photoreceptor outer segment regeneration and restoration of the ellipsoid zone were only identified in AAV-treated eyes, with therapeutic effect seen as early as 1-month post-injection *(p=0.001;* **Fig.2***)*. In *C57BL6J* mice, there was no difference in ERG assessments (A-wave, *p=0.65*; B-wave, *p=0.47*; Cone responses, *p=0.09;* **Fig.1**) or photoreceptor thickness measurements at 6 months between AAV and PBS-injected eyes *(p=0.19;* **Fig.2***)*. **Conclusion** These data provide proof-of-principle of the efficacy and safety of *CDHR1* gene therapy in a pre-clinical model of *CDHR1*-associated retinal degeneration. Rod and cone rescue occur through prevention of photoreceptor cell death and regeneration of photoreceptor outer segments. A follow-on clinical trial in patients with *CDHR1*-associated retinal degeneration is warranted.



AAV Therapies for Neurological and Sensory Diseases

137. scAAV9 Gene Replacement Therapy for Epileptic SLC13A5 Deficiency

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SLC13A5 Deficiency is an autosomal recessive disorder. This severe and rare form of epileptic encephalopathy is due to mutations in the *SLC13A5* gene, which codes for a plasma membrane sodiumdependent citrate transporter. To date all tested mutations result in no or a much reduced amount of the citrate transported inside the cells. Affected children present with seizures beginning within a few days of birth that persist throughout life. They show difficulty with speech production, limited and slow motor progress and many never achieving independent walking. Currently there are no treatments for SLC13A5 deficiency that target the underlying cause of disease and patients require constant supervision and care. Anti-epileptic drugs have varying success even amongst siblings, and children can succumb to complications of the seizures. Gene replacement therapy represents a therapeutic option for SLC13A5 Deficiency. We developed a self-complementary vector encoding a codonoptimized human SLC13A5 gene (scAAV9/SLC13A5), the unaltered design of which could be appropriate for human use. This study aims to compare safety and efficacy of scAAV9/SLC13A5 delivered intrathecally-lumbar puncture (IT) or intra-cisterna magna (ICM) in SLC13A5 knockout (KO) mice and wild type (WT) littermates. KO mice do not have an overt phenotype; however, similarly to patients, they have increased extracellular citrate and

abnormalities in TCA intermediates. KO mice have low, but increased epileptic activity as compared to WT mice and continuous EEG recordings can detect seizure types ranging from myoclonic and focal and generalized convulsive, like those seen in patients. KO mice and WT littermates were treated with scAAV9/SLC13A5 via IT delivery or ICM delivery at 10-12 weeks of age. Mice were monitored for weight and survival. Blood was collected at baseline and then monthly after injection and ~5 months after treatment, mice received telemetry implants to record EEG and EMG activity over two 60 hour recording sessions. These mice were then tested for susceptibility to seizure induction by pentylenetetrazol (PTZ). In agreement with previous studies, at baseline, KO mice had increased plasma citrate levels as compared to WT littermates. At one-month post-treatment and beyond, KO mice treated with scAAV9/SLC13A5 had significantly decreased plasma citrate levels while KO mice treated with vehicle had sustained, high citrate levels. EEG assessments showed that vehicle treated KO mice had increased spike train activity and seizure frequency compared to their WT littermates and, importantly, that vector treatment reduced this epileptic activity with greater rescue achieved with ICM delivery than IT delivery. Using the Racine scale, SLC13A5 KO mice also had increased PTZ-induced seizure susceptibility, which was rescued to WT levels with ICM delivery of scAAV9/SLC13A5 and to a lesser extent with IT delivery. Additionally, the safety profile of the SLC13A5 vector was excellent with no adverse effects on weight, general activity or survival in KO or WT mice with either IT or ICM delivery. Overall, our preclinical results suggest that gene replacement therapy with scAAV9/SLC13A5 could provide a meaningful benefit to SLC13A5 patients.

Advances in Cellular and Immunotherapies?

138. Dissecting the Transcriptional and Epigenetic Landscape of hiPSC-Derived Neural Stem and Progenitor Cells: Implications for Cell Therapy Approaches

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Human induced pluripotent stem cell (hiPSC)-derived neural stem/progenitor cells (hiPS-NSPCs) are a promising cell source for cell therapy approaches and for the development of novel *ex vivo* gene therapy strategies to target neurodegenerative disorders with unmet clinical need. We have shown that intracerebral transplantation of genetically modified hiPS-NSPCs provides full rescue of the enzymatic deficit in the murine model of metachromatic leukodystrophy (MLD), a fatal demyelinating disease caused by genetic mutations of the arylsulfatase A (ARSA) enzyme. The optimization of hiPS-NSPC production (purity, homogeneity) and a comprehensive safety assessment are mandatory in view of prospective clinical application. In this study, we evaluated the transcriptional and epigenetic mechanisms underlying the hiPSC to neural commitment and complemented these data with phenotypic and functional studies in order to define a comprehensive hiPS-NSPC signature and safety profile. Computational integration of RNA-seq and ChIP-seq data revealed a strong downregulation of pathways regulating pluripotency, cell cycle, and cancer-related processes with the concomitant appearance of a distinct "neural signature" in hiPS-NSPCs. Interestingly, we highlighted a dramatic change in the usage of cell-specific enhancers and super-enhancers during hiPSC neural commitment, suggesting its major role in the generation and maintenance of hiPS-NSPCs. Differences in the transcriptomic and epigenetic profiles between hiPS-NSPCs and human fetal NSCs (hfNSC, used as reference) could be ascribed to culture conditions, regionalization pattern, and differentiation potential, with no major signs of abnormal differentiation and activation/misregulation of cancer-related pathways attributable to a pluripotent "memory". Single cell RNA-seq analyses confirmed that hiPS-NSPCs are a heterogeneous cell population mainly composed by radial glial stem cells and committed neuronal and glia progenitors, and do not include pluripotent cells. Indeed, upon intraventricular transplantation in neonatal immunodeficient mice hiPS-NSPCs engrafted and widely dispersed in the brain parenchyma, migrating along the rostro-causal axis and differentiating in mature neurons and glial cells, with no sign of abnormal cell proliferation up to 12 months after treatment.This study contributes to the development of strategies for increasing safety and efficiency of hiPS-NSPC transplantation approaches for the treatment of neurodegenerative and demyelinating disorders.

139. Receptor Engineered TRuC Tregs Maintain a Regulatory Phenotype and Are Suppressive in a Murine Model of Hemophilia A

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The most serious complication to clotting factor VIII (FVIII) replacement therapy in hemophilia remains the development of inhibitory antibodies (inhibitors), which occurs in a significant proportion of patients with severe disease. Tolerance to exogenously administered FVIII is strongly dependent on regulatory T cells (Tregs) and it is expected that "redirecting" antigen specificity by engineering Tregs with synthetic receptors will overcome low inherent precursor frequencies and simultaneously augment suppressive functions in Treg cellular therapy. Our preliminary data indicate that employing a high affinity chimeric antigen receptor

(CAR) molecule paradoxically results in heightened signaling and loss of suppressive activity in transduced Tregs. Therefore, we applied an alternative approach, which is to complex a FVIII antibody based single-chain variable fragment (scFv) to the N-terminus of murine CD3ε subunit of TCR complex. The resulting TCR fusion construct (TRuC) Treg is able to recapitulate TCR based signaling in an MHC-independent fashion. We initially confirmed TCR dependent surface expression of TRuCs by flow cytometry using a FVIII binding assay, where co-transduction of FVIII-TRuC with TCR and CD3 components resulted in transportation of the engineered receptor to the plasma membrane surface in 5KCα^{-}β^{-} cells (which lack TCRα and β) and in human HEK-293 cells (which lack TCR and CD3). FVIII stimulation of TRuC-Tregs *in vitro* led to upregulation of CD69, Ki67, CD28, FoxP3, and a 5-fold increase in CTLA4 expression (p=0.0001, 1-way ANOVA). TRuC Tregs secreted significantly lower levels of cytokines IL-2, IL-4, IL-17, IL-10 and IFNγ as compared to CAR Tregs. This was confirmed by intracellular cytokine staining. Phospho-flow cytometry and western blotting confirmed dampened expression of critical signaling proteins pAKT S473, pERK and pS6 in TRuC Tregs, which was similar to levels observed on triggering the endogenous TCR in Tregs with anti-CD3/28 microbeads. These results confirm that FVIII stimulated TRuC Tregs maintain a Treg phenotype. Importantly, FVIII stimulation and TRuC signaling did not result in a loss of lineage stability in transduced Tregs, as TRuC Tregs from FoxP3-GFP mice retained FoxP3 expression in vivo (92.75±0.3% GFP^{+} cells). We next investigated whether TRuC Treg functional responses was sufficient to maintain a suppressive phenotype *in vitro* and *in vivo*. FVIII TRuC Tregs were able to suppress the *in vitro* proliferation of TRuC Tconv responders when stimulated with low dose (0.1 IU/mL) BDD-FVIII, without any requirement for antigen presentation. *In vivo*, naïve BALB/c *F8*e16$^{-/-}$ recipient mice were infused with 5x10^{5} sorted TRuC Tregs or polyclonal thymic derived (t)Tregs (5x10^{5} or 1x10^{6}) followed by 8 weekly IV injections of 1.5 IU BDDFVIII. FVIII TRuC Tregs were more effective at suppressing inhibitor formation as compared to polyclonal tTregs. 7 out of 8 animals in the TRuC Treg group did not develop detectable inhibitors (0.23±0.23 BU/ mL) at 4 weeks compared 8 out of 8 in the control group (69.42±33.99 BU/mL). At 8 weeks, control mice had a mean inhibitor titer of 151.4±48.6 BU/mL compared to 15.4±10.4 BU/mL in the TRuC Treg group. αFVIII IgG1 titers were also significantly lower in the TRuC Treg group (5238±3862 ng/mL) compared to the 5x10^{5} (28429±3862 ng/mL, p=0.0042) or 1x10^{6} (21821±8020 ng/mL, p=0.0438) tTreg groups at 8 weeks, suggesting a more sustained tolerogenic effect. In conclusion, this study suggests that adoptive cellular therapy with antigen specific engineered TRuC Tregs is able to suppress antibody formation against the soluble therapeutic protein FVIII in an MHC-independent manner, in spite of persisting only transiently in vivo (~7 days). More studies are required on regulating activation thresholds to maintain optimal suppressive function in engineered Tregs.

140. Functional Benefit of Mitochondrially Augmented HSPCs: Improved Engraftment and Alterations in Immune Cell Differentiation

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mtDNA associated disorders (either deletions, mutations or depletion) lead to multisystemic disease, often severe at a young age, with no disease-modifying therapies. In mitochondrial augmentation therapy (MAT), hematopoietic stem and progenitor cells (HSPCs) are enriched with healthy donor mitochondria ex vivo. This process allows cells to import mitochondria harboring full length mtDNA, resulting in increased mitochondrial content and improved function. HSPCs were chosen as recipient cells for MAT due to their demonstrated ability to alleviate systemic (hematological and non-hematological) pathologies. We demonstrated that mitochondrial augmentation of HSPCs is dose-dependent and confers functional benefit in both healthy donor and patient-derived HSPCs. We showed that HSPCs can be enriched with up to 34.9% exogenous mtDNA, resulting in increased oxygen consumption rate. To assess the potential effects of mitochondrial augmentation on human HSPCs, we used a humanized NSGS mouse transplanted with cord-blood derived CD34+ cells from a patient with Pearson Syndrome (PS), a mtDNA deletion syndrome. In this non-conditioned model, mitochondrially augmented CD34+ cells had improved long-term engraftment in the NSGS mice, as confirmed by flow cytometry and dPCR. Multi-lineage hematopoietic potential of engrafted cells was demonstrated, and animals which received mitochondrially enriched cells had significantly higher percentages of human CD3+ T cells. To investigate persistence in an immunocompetent animal, we developed a mouse model in which all nuclei were labelled with red fluorescence (Tomato) and all mitochondria were labelled with green fluorescence (Dendra). Recipient Polg mice had a point mutation in the mitochondrial DNA polymerase gene, leading to accumulation of mtDNA mutations. We were able to show long term persistence of daughter cells without

preconditioning treatment. Continuous in vivo transfer of exogenous mitochondria to recipient host hematopoietic cells was observed and persisted up to 4.5 months post transplantation, the last time point tested. Cells of both myeloid and lymphoid lienage were recipients of mitochondria in the peripheral blood. Taken together, these data provide evidence supporting feasibility of augmentation of human HPSCs and for the potential of MAT as a therapeutic modality for the treatment of mitochondrial disorders.

141. Memory Enriched Epstein-Barr Virus {EBV} Specific T-Cells with Broader Target Antigen Repertoire for the Treatment of EBV+ Malignancies

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Almost 40 % Hodgkin's and Non-Hodgkin Lymphoma {HL/NHL} patients carry the EBV genome in a type 2 latency state in which 4 viral type-2 latency antigens {T2-Ag} are expressed. T2-Ag-specific T-cells from lymphoma patients are difficult to expand, likely because they are rendered anergic in the tumor microenvironment, and circulate with low frequency. In a clinical trial-NCT01555892 using patient-derived, EBV-specific T cells {EBVSTs} to target these tumors, ~25% of EBVST lines failed our manufacturing criteria due to lack of antigen specificity or failure to grow. Even in successfully expanded lines, over 50% recognized only 1 or 2 antigens, which could lead to tumor escape by antigen modulation. We ***hypothesized*** that low antigen specificity could result from the expansion of non-specific bystander cells in our cultures, and that enrichment of memory T-cells prior to culture as well as stimulation with additional viral tumor antigens could improve their potency. To enrich for memory T-cells, we **depleted the CD45RA+ fraction** of PBMCs that contains naïve T-cells, T-regs and NK-cells prior to EBVSTs generation. To broaden the tumor-specific T-cell repertoire, we evaluated EBV lytic cycle antigen expression in HL biopsies and combined EBV lytic antigen and T2-Ag peptide libraries to generate **broad repertoire {BR}-EBVSTs** with specificity to both in γ-IFN ELIspot assays. Depletion of CD45RA+ cells prior to EBVSTs generation increased the frequency of T2-Ag specific T-cells by 2-10 fold, and enhanced proliferation and cytotoxicity compared to EBVSTs generated from unfractionated PBMCs. Most importantly, we restored the ability of patient EBVSTs to respond to EBV T2-Ags. This unexpected finding suggested that CD45RA+ cells are capable of inhibiting the reactivation and expansion of T2-Ag specific T-cells. In support of our proposal to target both T2- and lytic antigens, we identified both T2-latent and lytic cycle transcripts in HL biopsy samples and demonstrated that BR-EBVSTs could be generated by stimulation with a combination of T2- and lytic-cycle antigens. Following adoptive transfer into an EBV+ murine xenograft model, autologous BR-EBVSTs cleared tumor more rapidly {average by

day 15} than T2-EBVSTs {average by day 30} and better limited metastatic spread. Notably human GM-CSF and IFN-γ serum levels were 2-fold or higher in mice treated with BR-EBVSTs, which should create a more pro-inflammatory tumor milieu leading to increased tumor killing and epitope spreading in lymphoma patients. **BR-EBVSTs from memory enriched CD45RA+ subset depleted PBMCs** are now under evaluation in our amended clinical trial. We generated EBVSTs with high antigen specificity from 7/7 lymphoma patients and treated five, including the first patient whose EBVSTs had failed manufacturing using the previous protocol {Fig 1.}

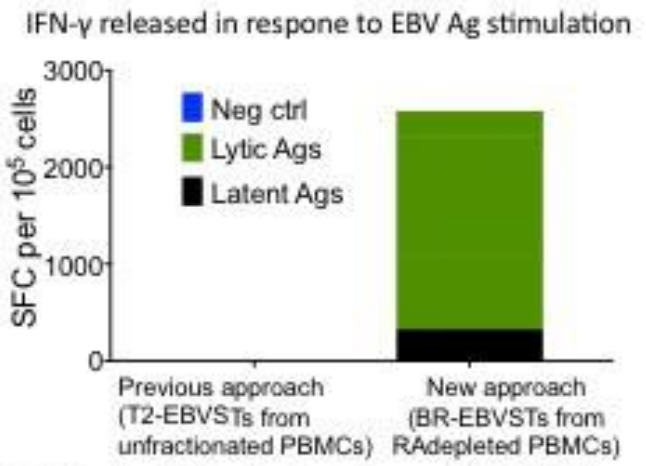

Fig 1.) Bar Graph illustrating the antigen specificity of EBVST lines generated for patient 1 by two different approaches. Y-axis represents the Spot Forming Cells (SFC) as a measure of number of cells that secreted IFN-γ in response to EBV antigen stimulation.

We have observed an increase in the frequency of T-cells recognizing both T2- and lytic antigens in patient PBMCs post-infusion for up to 3 months. Long-term follow-up and comparison with patients who received T2-EBVSTs from unfractionated PBMCs will determine if these changes indeed produced a more potent clinical product.

142. Non-Viral Engineering of CAR-NK Cells Using the *TC Buster* Transposon System™

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Immunotherapy with T cells and NK cells modified with viral vectors to express a chimeric antigen receptor (CAR) has shown remarkable efficacy in clinical trials. However, viral vectors are limited in their cargo size, carry the risk of insertional mutagenesis, and large-scale manufacturing for clinical use can be cost-prohibitive. Thus, CAR delivery via DNA transposon engineering has been pursued as an alternative due to convenient and cost-effective production and a better safety profile. Engineering via transposition is accomplished using a two-component system: a plasmid containing a gene expression cassette flanked by inverted terminal repeats (ITRs) and a transposase enzyme that binds to the ITRs and integrates the transposon into the genome. Here, we sought to use the newly developed *TC Buster*™ (Bio-Techne) transposon system to deliver a transposon containing a CD19-CARDHFR-EGFP expression cassette (3.7 kb transposon, figure 1A) to primary human peripheral blood (PB) NK cells. However, the use of transposons in NK cells has been very limited due to DNA toxicity and induction of a type I interferon response. Thus, we optimized methods to avoid this, including using DNase in recovery media and delivery via Nanoplasmid vectors which have a small (<500 bp) backbone, high supercoiling, and are regulatory compliant. We optimized activation, electroporation, recovery, and expansion

conditions to achieve 49.6% (±4.64%) integration efficiency using an evolved hyperactive *TC Buster*™ transposase (Hyp-TCB) (Figure 1B). Our cargo also contained a mutant dihydrofolate reductase (DHFR) which allowed us to select for stable transposon integration using methotrexate (MTX). Our optimized protocol achieves manufacturing in 20 days and results in 99.2% (±0.5%) CAR+ NK cells expanded 837.9-fold (±88.6) from input (Figure 1C, 1D). We tested CAR-NK cells in functional assays against CD19-expressing Raji cell lines. CAR-NK cells produced significantly more IFNγ and TNFα than CAR-negative NK cells when co-cultured with Raji (Figure 2A, 2B). CAR-NK cells also expressed significantly more CD107a on their surface, a marker of degranulation (Figure 2C). In killing assays, CD19-CAR-NK cells killed over 90% of Raji cells in 24 hours at effector-to-target ratios as low as 1:3 (Figure 2D). Our work provides a platform for robust delivery of multicistronic, large cargo via transposition to primary human PB NK cells. We are currently using this platform to deliver larger cargo of interest that far exceeds the carrying capacity of viral vectors. We demonstrate that CAR-NK cells can be enriched using MTX selection, while maintaining high viability and function, and they can be expanded to clinically relevant numbers in a matter of weeks. In recent experiments we have demonstrated successful delivery of *TC Buster*™ transposons for large cargo integration with CRISPR/Cas9 for targeted gene knockout in a single electroporation event. This non-viral approach for multiplex editing of NK cells represents a versatile, safer, and more cost-effective option for the manufacture of CAR-NK cells compared to viral delivery.

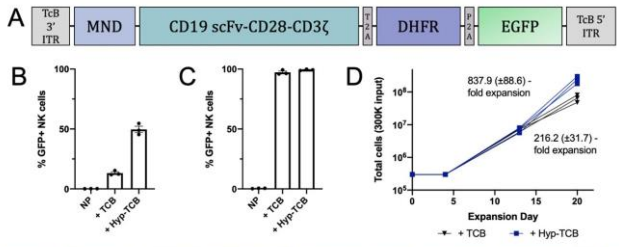

Figure 1. Delivery of a CD19-CAR-DHFR-GFP transposon using the evolved hyperactive TC Buster Transposon System. (A) The 3.7 kb transposon flanked by TC Buster ITRs, containing an MND promoter, CD19 CAR, mutant DHFR selection gene, and enhanced GFP. Elements are separated by 2A self-cleaving peptides. Transposon was delivered in a nanoplasmid (NP) backbone. (B) Primary human peripheral blood NK cells (n=3 human donors) were electroporated with the nanoplasmid (NP) alone or in combination with mRNA encoding either TC Buster (TCB) or the evolved hyperactive mutant TC Buster (Hyp-TCB). Two days after electroporation, NK cells were expanded with mbIL21-expressing feeder cells for 1 week. After expansion, GFP expression was measured by flow cytometry. (C) NK cells were expanded again with mbIL21-expressing feeder cells for one week in media containing 250 nM methotrexate (MTX) and then GFP expression was measured by flow cytometry. (D) Fold expansion was calculated over the course of the experiment.

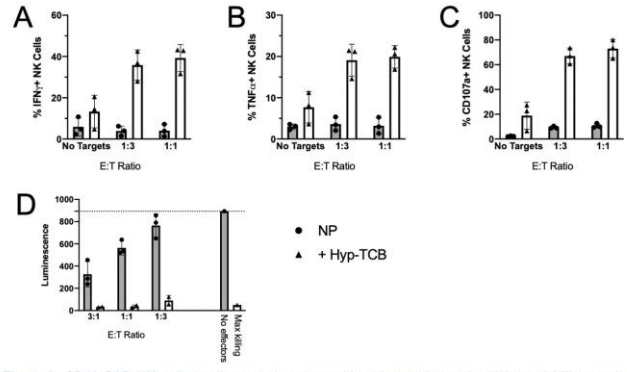

Figure 2. CD19-CAR-NK cells engineered via transposition show enhanced activity and killing against CD19+ target cells. MTX-selected NK cells (n=3 human donors) expressing CD19-CAR were co-cultured with luciferase-expressing, CD19-positive Raji target cells. (A-C) After co-culture, NK cells were analyzed by flow cytometry for expression of IFNγ, TNFα, and CD107a. (D) Target cell killing was measured by luciferase expression after co-culture.

143. Adoptively Transferred, *In Vitro* Generated Alloantigen-Specific Type 1 Regulatory T (Tr1) Cells Persist Long-Term *In Vivo*

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Allogeneic, HLA-mismatched hematopoietic stem cell transplantation (allo-HSCT) can be a life-saving treatment for many high-risk hematological malignancies, as well as for certain non-malignant disorders. However, the largest obstacle to wide-spread use of allo-HSCT is its frequent and life-threatening side-effect, graft-vs-host disease (GvHD). GvHD is caused by HSCT donor-derived effector T (Teff) cells that attack patient's HLA-mismatched healthy tissues. GvHD can be treated, but all standard-of-care treatments also induce generalized immunosuppression, increasing the risk of relapse, infection, and death. To alleviate GvHD without causing generalized immunosuppression, we aim to leverage type 1 regulatory T (Tr1) cells. Tr1 cells are inducible, IL-10+FOXP3- subset of regulatory T cells that was shown to correlate with tolerance in allo-HSCT patients, and prevent GvHD in mice. Tr1 cells suppress the activity of Teff cells, which cause GvHD *in vivo*, through soluble IL-10 and co-inhibitory surface receptors. We have generated alloantigen-specific Tr1 cells by co-culturing HSC donor-derived CD4 T cells with patient-derived tolerogenic dendritic cells, which present patient alloantigens and induce

differentiation of CD4 T cells into Tr1 cells. During the differentiation, Tr1 cells clonally expand, developing a highly restricted TCR repertoire. The final Tr1-enriched cell product, called T-allo10, is anergic and suppresses Teff cell proliferation specifically in response to alloantigens, but not irrelevant antigens (Cepika AM, Chen PP et al, J ImmunoTher Cancer 2020; Vol. 8, S3:146). T-allo10 cells are being tested for safety in a phase I clinical trial in children and young adults with hematological malignancies undergoing allo-HSCT (ClinicalTrial.gov ID: NCT03198234). In this trial, T-allo10 cells are adoptively transferred one day before allo-HSCT, and patients are immunomonitored in 19 time points from day 0 to 360. One year-post treatment, the first three patients

are alive, have met the safety criteria, and are GvHD and cancer-free (Agarwal R et al, Biol Blood Marrow Transplant 2020; Vol. 26 (3): S272-S273). To demonstrate the long-term survival of adoptively transferred Tr1 cells in these first three patients, we FACS-sorted the highly purified Tr1 cells from patient's T-allo10 cell product, and compared their TCR repertoire to non-Tr1 cells from the same product, parental CD4 T cells, and control Teff cells. We determined the top 20 most highly enriched TCR clonotypes of Tr1 cells, and then compared this data to the patient whole blood TCR repertoire in all 19 time-points. Using this method as complementary to peripheral Tr1 cell detection by flow cytometry, we demonstrate that Tr1 cells persist in patients for up to 1-year post adoptive transfer. This implies that Tr1 cells represent a durable cell therapy option to modulate antigen-specific tolerance.

144. Endothelial Progenitor Cells Engineered to Overexpress Endothelial NO-Synthase May Improve Infarct Healing: Results from the Enhanced Angiogenic Cell Therapy - Acute Myocardial Infarction (ENACT-AMI) Trial

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Introduction: Despite modern reperfusion therapies for acute myocardial infarction (AMI), many patients are left with a significant area of injury leading to scar formation that contributes to a dysfunctional myocardium and the development of heart failure. The goal of the Enhanced Angiogenic Cell Therapy-AMI (ENACTAMI) trial was to determine whether intracoronary delivery of early outgrowth endothelial progenitor cells (EPCs) engineered to overexpress endothelial NO-synthase (eNOS) would improve global left ventricular ejection fraction (LVEF) (primary outcome) and infarct size as assessed by cardiac MRI (CMR) in patients with LAD territory ST elevation myocardial infarction treated with evidence-based therapies. **Methods**: ENACT-AMI (NCT00936819) was a double-blind placebocontrolled trial in which participants were randomized to one of 3 arms:

1) saline placebo; 2) EPCs; or 3) eNOS-transfected EPCs. EPCs were derived from circulating mononuclear cells by 7-8 days of differential culture on fibronectin in EGM supplemented with endothelial growth factors using fully sourced GMP-compatible reagents. Transfection was performed using a plasmid DNA vector (pVax) containing the human eNOS sequence combined with JetPEI (Polyplus). Target sample size was 100 participants. Groups were compared using analysis of covariance (ANCOVA). **Results**: The trial was terminated due to slow recruitment after 47 patients were enrolled at three Canadian sites over six years (n=18 placebo, n=15 EPCs; n=14 eNOS-transfected EPCs). The groups were comparable with respect to demographic variables including age (56.1±9.7 years), cardiac risk factors, pre-existing cardiac disease, peak CK and troponin values and baseline LVEF by cardiac magnetic resonance imaging (40.7±9.3). Intracoronary cell delivery (20M cells; 20±5 days post-AMI) was well tolerated. At 6 months, there were no significant differences in the primary endpoint of LVEF between groups (mean difference between the average of the two EPC groups vs. placebo: 0.5% [95% Confidence Interval (CI) 2.9% to 3.9%). The secondary outcome of left ventricular infarct mass indexed to LV size at 6 months demonstrated no significant difference between the average of the two EPC groups versus placebo (p=0.72); however, a significant difference was seen in those receiving eNOS-transfected EPCs compared to EPCs (-6.6; CI -12.0 to -1.1, p=0.02). Only four major cardiovascular events were observed over an average follow up of 4.1±1.6 years, and these were equally distributed across groups. **Conclusions**: While there were no significant differences in LVEF, the results of the ENACT-AMI trial suggest that intracoronary delivery of gene-enhanced EPCs reduced infarct size and LV diastolic diameter in patients with large anterior wall AMI consistent with improved scar healing. These findings have important implications for remodelling and require confirmation in larger clinical trials.

CAR-Based Cancer Gene Therapy

145. Chimeric Antigen Receptor Macrophages (CAR-M) Induce Anti-Tumor Immunity and Synergize with Immune Checkpoint Inhibitors in Pre-Clinical Solid Tumor Models

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Despite the remarkable efficacy achieved by CAR-T cell therapy in hematologic malignancies, translating these results for solid tumors remains challenging. We previously developed human CAR-M and demonstrated that adoptive cell transfer of CAR-M into xenograft models of human cancer controls tumor progression and improves overall survival[1]. Given that CAR-M are professional antigen presenting cells, we established a fully immunocompetent syngeneic mouse model to evaluate the interaction of CAR-M with the tumor microenvironment (TME) and the potential for induction of a systemic anti-tumor immune response. Murine bone marrowderived CAR-expressing macrophages (muCAR-M) were efficiently engineered to express an anti-huHER2 CAR using the chimeric adenoviral vector Ad5f35. In addition to efficient gene delivery,

Ad5f35 transduction promoted a pro-inflammatory (M1) phenotype in murine macrophages. MuCAR-M, but not control untransduced (UTD) macrophages, specifically phagocytosed HER2+ target cancer cell lines and killed HER2-expressing murine CT26 colorectal and human AU-565 breast cancer cells in a dose-dependent manner. Moreover, CAR-M induced MHC-I and MHC-II expression on tumor cells and cross-presented tumor-associated antigens (TAA) resulting in CD8+ T cell activation. To evaluate muCAR-M in an immunocompetent *in vivo* setting, BALB/c mice were engrafted with subcutaneous CT26HER2+ tumors and treated with HER2-CAR or UTD macrophages. CAR-M treated mice showed significant tumor control and improved survival compared to control groups. Analysis of the TME showed increased intratumoral immune infiltration as well as an increase in T cells reactive to the CT26 MHC-I antigen gp70, indicating enhanced epitope spreading. Mice that achieved complete responses (CRs) after CAR-M therapy were protected against antigen-negative relapse in a HER2- CT26 (CT26-Wt) rechallenge model. This anti-tumor immune response was CD3+ T cell-mediated and suggested induction of long-term memory against TAA. To evaluate the systemic anti-tumor immune response, we simultaneously engrafted BALB/c mice with CT26-HER2+ and CT26-Wt tumors on opposite flanks and treated mice with local administration of CAR-M to the HER2+ tumors. After CAR-M treatment, 75% of mice cleared their CT26-HER2+ tumors and the growth rate of the contralateral CT26-Wt tumors was significantly reduced, demonstrating an abscopal effect. Given the impact of CAR-M on the endogenous adaptive immune system, we evaluated the combination of CAR-M with PD1 checkpoint inhibitor therapy in the CT26-HER2 model, which is resistant to anti-PD1 monotherapy, and found that the combination further improved tumor control and overall survival. These results demonstrate that CAR-M reprogram the TME, induce epitope spreading, and orchestrate a systemic immune response against solid tumors. Moreover, our findings provide rationale for the combination of CAR-M with immune checkpoint inhibitors. The antiHER2 CAR-M, CT-0508, is under evaluation in a phase I clinical trial for patients with HER2 overexpressing solid tumors (NCT04660929).

1. Klichinsky M, Ruella M, Shestova O, et al. Human chimeric antigen receptor macrophages for cancer immunotherapy. Nat Biotechnol. 2020;38(8):947-953.

146. Bivalent CD19/CD20-Specific CAR T Cells with 4-1BB and Mutated CD28 Co-Stimulatory Domains Show Enhanced Function

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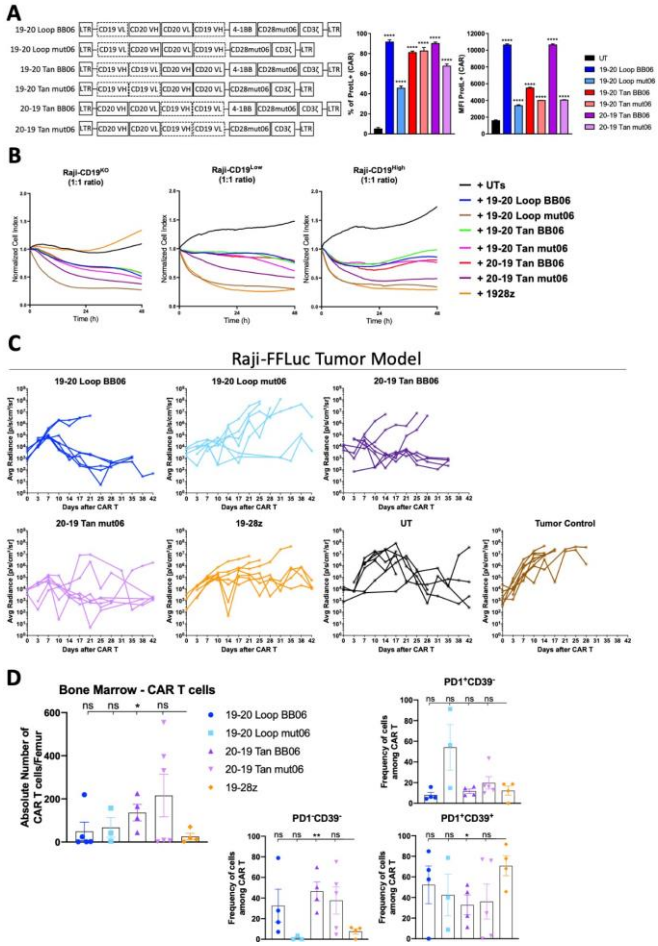
Despite the high response rates of CD19 CAR T cell therapy, the loss or downregulation of CD19 has been reported as a common mechanism of tumor resistance. Current strategies focused on overcoming antigen escape include the use of CAR T cells able to recognize more than one target antigen. Additionally, the choice of co-stimulatory domains impact CAR T cell expansion, persistence and antitumor function. Our recent study found that mutating the CD28 endodomain to retain signaling only through the PYAP motif (mut06) rendered enhanced antitumor activity to CAR T cells, which was in part due to resistance to T cell exhaustion (Boucher et al. 2020). For this work we developed bi-specific constructs encoding for single bivalent CAR molecules able to recognize both human CD19 (FMC63) and CD20 (Leu16) in a loop or tandem configuration. To take advantage of the bi-specificity and the enhanced co-stimulatory domain we developed second generation bivalent CARs with mutated CD28 (mut06) and third generation CARs containing both 4-1BB and mut06 (BB06). We determined efficient surface CAR expression in all constructs and increased central memory phenotype in third generation BB06 CARs (Figure 1A). We detected secretion of significant levels of IFNγ, IL-2, TNFα, and minimal levels of IL-6 upon engagement to either CD19 or CD20 with the highest levels observed when both antigens were present. We determined in vitro cytotoxicity by a real-time assay (RTCA) against either antigen alone or both and observed significant lytic effect of all constructs with second generations (mut06) CARs showing a faster response (Figure 1B). We then analyzed in vivo antitumor function of these bivalent CAR T cells against a xenograft model of Raji-FFLuc. Third generation CAR T cells with mutated CD28 (BB06) showed a more effective tumor growth control compared to mono-specific anti-CD19 with unmutated CD28 co-stimulation (1928z)(Figure 1C). Importantly, these BB06 CAR T cells showed increased persistence in the mice at day 45 post-inoculation compared to 1928z. Furthermore, BB06 CAR T cells displayed significantly lower frequency of cells expressing the exhaustion-associated markers PD1, TIM3 and CD39 compared to 1928z and second-generation mut06 cells (Figure 1D). In a subsequent in vivo experiment using Raji cells only expressing CD20 (Raji-CD19KO) we were able to validate our previous results showing 20-19 tandem BB06 as the most effective construct against this CD19KO model. To date our results demonstrate that these bi-specific CAR T cells are able to recognize and trigger an effector function upon engagement of CD19 and/or CD20 and, most importantly that the combination of 4-1BB and this new mutated form of CD28 as co-stimulation enhance the antitumor function of cells. This work supports the concept that CAR T cell therapies can be improved by optimizing their design, including this dual antigen-binding domain and modified co-stimulatory motifs.



147. Combining IAP Inhibitors with CAR T Cell Therapy to Treat Glioblastoma

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Antigen heterogeneity is one of the major obstacles to successful chimeric antigen receptor (CAR) T cell treatment in solid tumors including glioblastoma, as antigen-negative tumor cells could escape from the CAR-T cells. To address this issue and improve the efficacy of CAR-T cell therapy for glioblastoma, we are developing an approach that combines IAP inhibitors, a new class of small molecules, with CAR-T cells to treat glioblastoma. IAP inhibitors can induce the degradation of inhibitor of apoptosis (IAP) proteins. By antagonizing IAP proteins, IAP inhibitors could sensitize glioblastoma cells, including antigen-negative cells, to apoptosis induced by CAR-T cell-derived tumor necrosis factor (TNF). Meanwhile, IAP inhibitors may promote CAR-T cell survival, proliferation and cytokine production including TNF via NF-kB inducing kinase (NIK)-mediated NF-κB pathways. We demonstrate that the IAP inhibitor Birinapant, currently in early phase clinical studies, induces the degradation of cellular IAP1 (c-IAP1) in the human glioblastoma cell line M059K, and exogenous TNF and Birinapant synergistically kill M059K via apoptosis. We further show that while CAR-T cells targeting glioblastoma antigen epidermal growth factor receptor variant III (EGFRvIII) do not directly kill M059K parental cells lacking EGFRvIII expression, Birinapant enhances the "by-stander" cell death of M059K parental cells when co-cultured with M059K cells transduced with EGFRvIII and the anti-EGFRvIII CAR-T cells, which express TNF when stimulated. Furthermore, our data show that conditioned medium from activated CAR-T cells mediates M059K cell death in the presence of Birinapant, and this death is partially blocked by TNF neutralization by infliximab, supporting the role of TNF in the mechanism of by-stander killing. In addition, we investigated how the IAP inhibitor Birinapant affects CAR-T cell functions. Exploiting an *ex vivo* repetitive stimulation assay, we show that Birinipant promotes CAR-T cell proliferation as measured by a higher fraction of cells in S-phase and also enhances TNF production by the CAR-T cells with prolonged antigen stimulation. These effects are associated with the activation of both the canonical and non-canonical NF-kB pathways. Glioblastoma xenograft models with heterogeneous tumor antigen expression are currently being developed to evaluate the efficacy of combining CAR-T cells with Birinipant *in vivo* in order to prevent escape due to antigen heterogeneity. In conclusion, our results support the potential for combining IAP inhibitors such as Birinipant as a novel approach to addressing antigen heterogeneity and tumor escape that represent a major hurdle to CAR-T cell-based immunotherapies.

148. B-CLL-Mediated Insufficient Activation Is CAR-Independent

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Background: Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in the western world, accounting for nearly 1/3rd of adult leukemia diagnoses. While standard-of-care chemoimmunotherapies are initially successful in treating this disease, the lack of curative therapeutic options means that most patients will ultimately succumb to their disease. Chimeric Antigen Receptor (CAR) T cell therapy has proven effective, but only in a small subset of patients. Improving response rates to this therapy will provide muchneeded curative options for this patient population. **Findings:** We have previously shown that the inability of CLL cells to activate CAR T cells drives this lack of response. In a serial re-stimulation model, we observed defects in both CD19- and ROR1-directed CAR T cell proliferation, cytokine production, and cytotoxicity. Flow cytometry showed that CLL-stimulated CAR T cells maintained an un-activated profile, suggesting that CLL cells fail to stimulate CAR T cells rather than rendering them non-functional. We further showed that these defects were non-permanent and could be rescued by stimulating CAR T cells with an artificial antigen presenting cell (aAPC) with or without the presence of CLL cells. Immunophenotyping of our B-CLL biobank showed that the majority of CLL cells (18/20 patients, 90%) express the IL-